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COMMUNICATION

Novel synthetic baicalein derivatives caused apoptosis and activated AMP-activated protein kinase in human tumor cells†

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Studies on the anti-proliferative activities of novel baicalein derivatives demonstrated that compounds 8 and 9 were able to activate AMPK by enhancing the levels of phosphorylated AMPKα, and showed more potent anti-proliferative effects than baicalein and AICAR in A431, SK-OV-3, DU 145 and HeLa cells, suggesting an alternative therapeutic approach for benzyl baicalein in cancer therapy.

1. Introduction

The radix of *Scutellaria baicalensis* (or Huang Qin) possesses anti-inflammatory, anti-oxidative, and anti-viral activity, and is commonly studied for its role in the treatment of various kinds of cancers both *in vivo* and *in vitro*.[1–3] The major bioactive phytochemical flavones identified in *Scutellaria baicalensis* are baicalin (7-glucuronic acid, 5,6-dihydroxyflavone) and baicalein (5,6,7-trihydroxyfavone), which have potent anti-tumor activity.[4,5] Many laboratory studies on cancer cell lines *in vitro* and *in vivo* in mouse tumor models have shown that baicalin has effects in modulating the cell cycle, inducing cell apoptosis and inhibiting cell proliferation, as well as suppressing tumor growth and prolonging survival.[6–8] Studies have also shown that baicalein induced cell proliferation inhibition and apoptosis in bladder cancer cells[9] and human pancreatic cancer cells.[10] These data demonstrate that baicalin and baicalein have therapeutic potential against cancers.

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase, acting as a cellular fuel sensor that monitors the AMP/ATP ratio to maintain cellular homeostasis.[11] Several metabolic stresses, including hypoxia, exercise, and starvation, are able to activate AMPK *in vitro* and *in vivo*.[11,12] Emerging evidence has also suggested that AMPK could be a potential therapeutic target for cancer.[13,14] Activation of AMPK by drugs, such as 5-amino-4-imidazolecarboxamide riboside (AICAR, an AMP analog) or metformin (an oral hypoglycemiant agent used as first-line therapy for type 2 diabetes), inhibits proliferation and induces apoptosis of human cancer cells,[15,16] and produces anticancer effects in SCID mice[17] as well as in patients with advanced nonsmall cell lung cancer[18] and with breast cancer.[19] Emerging evidence suggested that activation of AMPK by natural polyphenols could help to prevent the development of numerous diseases, including cancers.[20] We have recently reported that baicalin can activate AMPK both *in vitro* and *in vivo*, suppress systemic inflammatory stress and protect against the development of hepatic steatosis and obesity-related disorders in diet-induced obese rats.[21] Subsequently we observed similar phenomena with the structurally related flavone luteolin, which is also a potential anti-cancer and anti-proliferative agent and able to activate AMPK in HepG2 hepatoma cells.[22] Considering the important role of AMPK in inducing cell cycle arrest and apoptosis, and inhibition of tumor cell growth,[13] the ability of these flavones, including baicalin, to mediate AMPK activity is likely involved in their anti-cancer effects and could contribute to a novel therapeutic mechanism behind the efficacy observed with these flavones. Metabolism data suggest the rapid conversion of baicalin to baicalein in the intestines.[23] Herein, we wish to report the discovery of a few novel baicalein derivatives that are able to activate AMPK and induce apoptosis in several human tumor cell lines.

2. Results and discussion

2.1 Chemistry

It has been noted from the literature that the most active flavones are often hydroxylated at the C-5 position.[24–26] We have also found in our preliminary study that baicalein derivatives with the hydroxyl group kept at the C-5 position and with C-6 or C-7 modification showed greater potency in anti-oxidative activity (data not shown). Evidence has shown that the anti-proliferative effect of the flavonoids can be enhanced by synergistic interaction with antioxidants, and there was a correlation between the *in vivo* anti-inflammatory activity and the *in vitro* anti-oxidative activities.[27] Therefore, in the design of novel baicalein derivatives (Fig. 1), we decided to keep the three ring scaffold of baicalein (rings A, B and C) unaltered to maintain the key biological activities of flavone baicalein[24,28] and focus on the substitution pattern at positions 6 and 7 of the A-ring.

The general method used for the synthesis of *O*-substituted flavone derivatives from baicalein is depicted in Schemes 1 and 2. Acetylation of baicalein with acetic anhydride was performed

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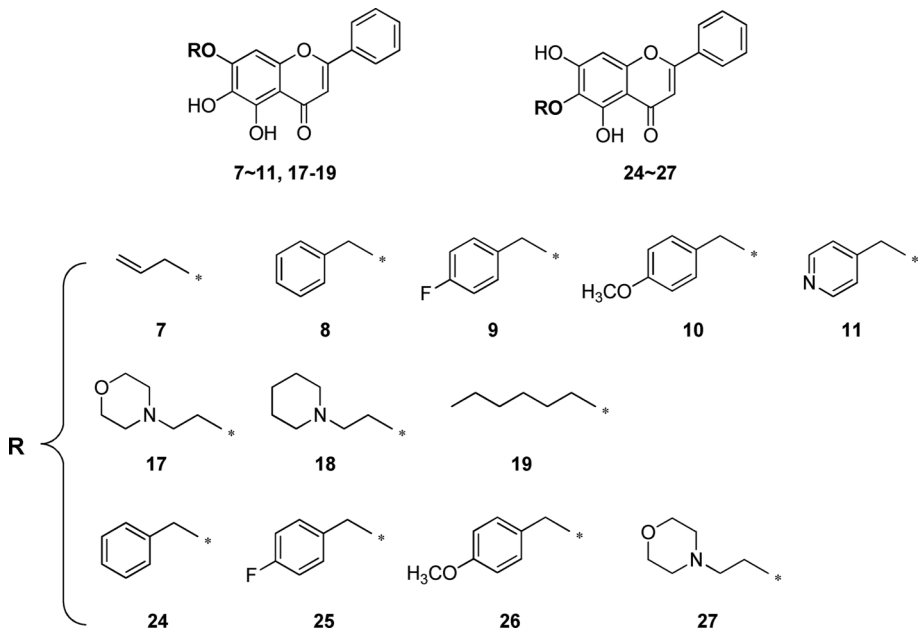
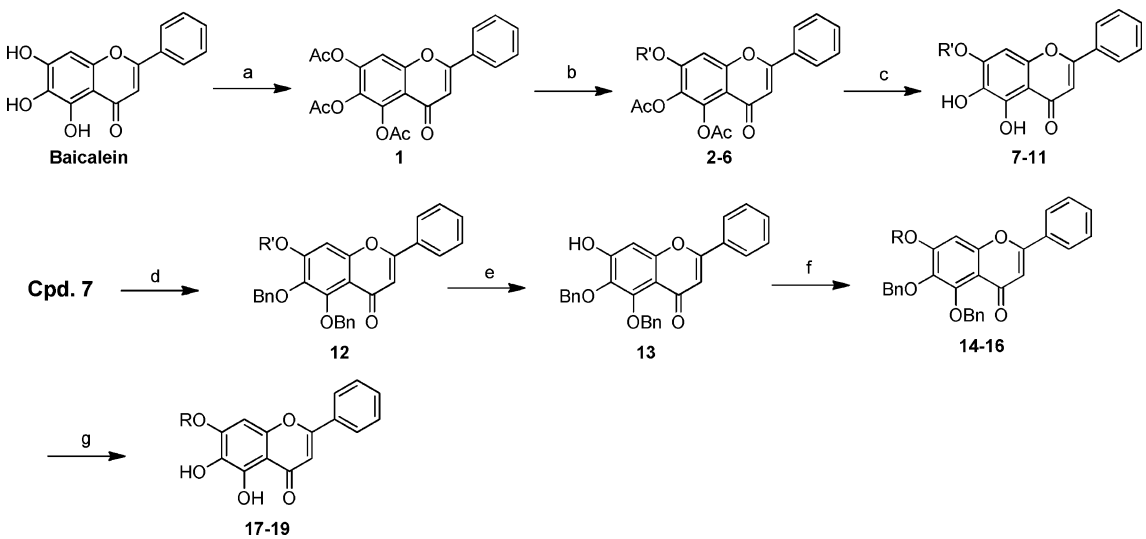


Fig. 1 Structures of synthetic baicalein derivatives.

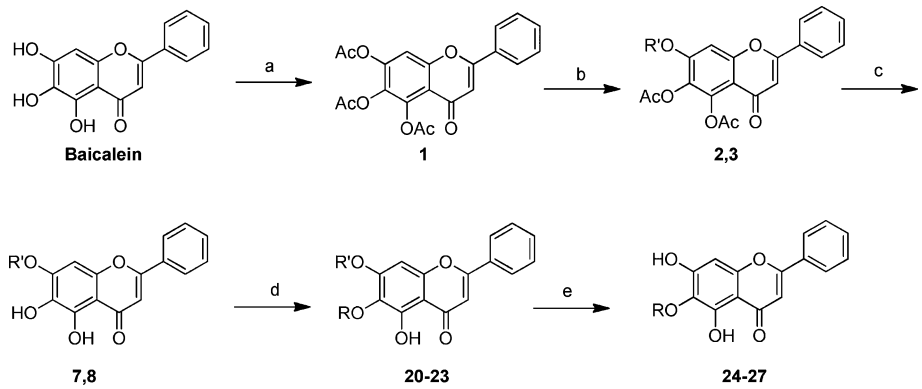


Scheme 1 Synthesis of 7-position substituted baicaleins. *Reagents and conditions*: (a) $(CH_3CO)_2O$, NaOAc, 75 °C; (b) allyl bromide/substituted benzyl bromides, K_2CO_3, acetone, reflux; (c) (i) aq. HCl, EtOH, reflux, (ii) aq. NaOH (5%), acetone; (d) for cpd. **7**: BnBr, K_2CO_3, acetone, reflux; (e) $Pd(PPh_3)_4$, $NaBH_4$, THF, rt; (f) RBr, Na_2CO_3, acetone, DMF, refiux; (g) Pd–C, H_2, THF, rt.

in NaOAc to give triacetylated derivative **1**. The triacetylated baicalein was treated with allyl bromide and substituted benzyl bromides in dry acetone with potassium carbonate to provide *O*-allyl and *O*-benzylated derivatives **2–6**. The deacetylation of *O*-allyl derivative **2** was performed with hydrochloric acid in ethanol to give compound **7**, whereas the hydrolysis of *O*-benzylated derivatives **3–6** was performed by the reaction with sodium hydroxide in acetone to give compounds **8–11**. The benzylation of compound **7** was performed with benzyl bromide in the presence of potassium carbonate in dry acetone to afford compound **12**. Since the hydroxyl function on the C-5 position of baicalein makes an intramolecular hydrogen bond with the 4-keto group, it is resistant to alkylation. The selective benzylation of compounds **7** and **8** was carried out under the same conditions as described above for the synthesis of compound **12** to afford the corresponding compounds **20–23** (Scheme 2). Through the reaction with different alkyl bromides using sodium carbonate as the base, deprotection of **12** with $Pd(PPh_3)_4$ and $NaBH_4$ in THF afforded compound **13**. Conversion of compounds **14–16** and **20–23** by catalytic hydrogenation gave the corresponding products **17–19** and **24–27**.

2.2 Biology

First, the anti-proliferative activity of all the synthetic compounds was evaluated on two human tumor cell lines, A431 (epidermoid carcinoma) and SK-OV-3 (ovary adenocarcinoma), by using the protein-binding dye sulforhodamine B (SRB) assays.[29] Among the 12 compounds tested, compounds **19** and **24** had cytotoxicity in



Scheme 2 Synthesis of 6-position substituted baicaleins. *Reagents and conditions*: (a) $(CH_3CO)_2O$, NaOAc, 75 °C; (b) (i) allyl bromide, K_2CO_3, acetone, reflux; (ii) BnBr, K_2CO_3, acetone, reflux; (c) (i) aq. HCl, EtOH, reflux; (ii) aq. NaOH (5%), acetone; (d) substituted benzyl bromide, K_2CO_3, KI, acetone, reflux; (e) (i) $Pd(PPh_3)_4$, $NaBH_4$, THF, rt; aq. NaOH, acetone; (ii) 10% Pd–C, H_2, THF, rt.

Table 1 Comparison of anti-proliferative activities of **8**, **9**, baicalein and AICAR

	IC_{50} (μM)[a]			
Cell line	**8**	**9**	Baicalein	AICAR
A431[b]	7.3 ± 1.1	6.4 ± 1.2	155	1257
SK-OV-3[c]	5.2 ± 1.1	3.7 ± 1.3	109	>2000
DU 145[d]	11.5 ± 1.2	6.9 ± 1.2	141	>2000
HeLa[e]	3.9 ± 1.1	1.5 ± 1.2	92	>3000

[a] As measured by the SRB assay after 48 h incubation of cells with drugs, results are expressed as IC_{50} (μM). [b] Human epidermoid carcinoma. [c] Human ovary adenocarcinoma. [d] Human prostate carcinoma. [e] Human cervical carcinoma.

A431 cells (cell growth inhibition ~55% at 25 μM) and in SK-OV-3 cells (cell growth inhibition ~56% at 25 μM), respectively, and only compounds **8** and **9** appeared to be cytotoxic in both of the tested tumor cells and displayed the most robust growth inhibitory effects among the synthesized baicalein derivatives (Fig. 5 in the ESI†). Furthermore, we compared the anti-proliferative activity of **8** and **9** with those of baicalein and AICAR, an activator of AMPK in 4 human tumor cell lines, A431, SK-OV-3, DU 145 (prostate carcinoma), and HeLa (cervical carcinoma). As shown in Fig. 2 and Table 1, compounds **8** and **9** inhibited the proliferation of the tested cells with average IC_{50} values of 3.9–11.5 μM and 1.5–6.9 μM, respectively, which were more potent than those of baicalein or AICAR, and this effect was most prominent in HeLa cells (Table 1).

Because we have shown that the flavones, including baicalin, were able to activate AMPK,[21,22] we next tested whether compounds **8** and **9** could also induce activation of AMPK by determining the phosphorylation status of AMPKα in the tested cells. AICAR, an AMP analogue and known AMPK activator, was used as a positive control. HeLa cells are deficient in LKB1 (one of upstream kinases of AMPK) and represent a natural "knock-out" cell line.[30] The results (Fig. 3) revealed that both compounds **8** and **9** were able to increase phosphorylation of AMPKα in the four tested cells. Other reports have shown that AICAR requires LKB1 to exert AMPK activation,[31] and that the loss of LKB1 causes the reduction of AICAR-mediated anti-proliferation effects on HeLa cells.[32] Interestingly, it was also shown in Fig. 3D that 1 mM AICAR was not able to stimulate phosphorylation of AMPKα in HeLa cells, leading to a small inhibition of cell growth, shown in Table 1, consistent with the report in ref. 32. However, both compounds **8** and **9** were still able to cause a significant increase in AMPKα phosphorylation in HeLa cells (Fig. 3D) and had even more potent anti-proliferative efficiency, with IC_{50} of 3.9 μM and 1.5 μM, respectively, compared to that in other cells (Table 1). In our primary study on HeLa cells we observed a similar result with baicalin (data not shown). Those findings suggested that phosphorylation and activation of AMPK in response to these synthetic flavones may not require the upstream kinase LKB1 and could be through alternative mechanisms that are different to AICAR.

Moreover, apoptosis was explored by flow cytometry (Table 2): compounds **8** and **9** similarly induced apoptosis in all four cell lines.

Table 2 Early and late apoptosis in A431, SK-OV-3, Du145, and HeLa cells treated with **8**, **9** and AICAR[a]

		% Cells	
Compd	Viability[b]	In early apoptosis[c]	In late apoptosis[d]
A431			
Control	94.3	1.3	4.1
8 (25 μM)	22.2	23.5	53.7
9 (25 μM)	19.4	19.9	60.4
AICAR (1 mM)	71.8	6.8	20.9
SK-OV-3			
Control	91.3	3.8	4.7
8 (25 μM)	70.7	15.5	13.5
9 (25 μM)	48.9	27.5	23.4
AICAR (1 mM)	71.9	9.5	18.4
DU 145			
Control	88.7	3.6	6.8
8 (25 μM)	58.4	15.4	24.9
9 (25 μM)	62.4	14.4	22.3
AICAR (1 mM)	71.9	9.5	18.6
HeLa			
Control	81.9	13.6	3.9
8 (25 μM)	11.4	36.2	49.9
9 (25 μM)	9.7	31.1	56.9
AICAR (1 mM)	73.8	14.0	10.7

[a] Expressed as the percentage of viable cells, cells in early and late apoptosis, measured by respective fluorescence intensities of Annexin V *vs.* PI. [b] Annexin V–/PI–. [c] Annexin V+/PI–. [d] Annexin V+/PI+.

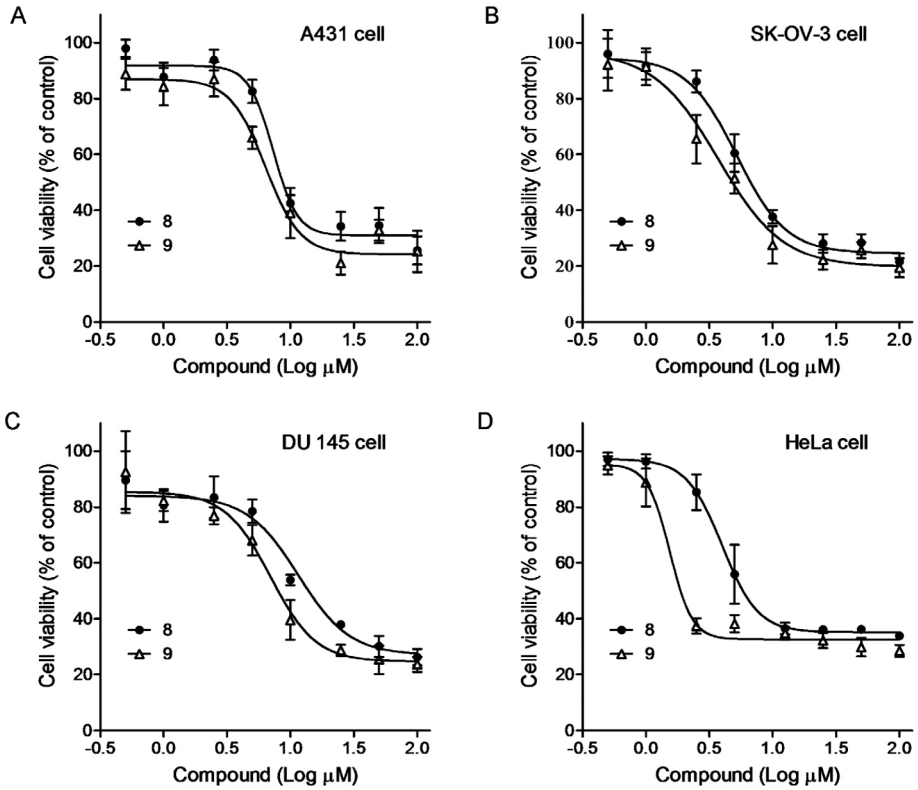


Fig. 2 Effects of compounds **8** and **9** on the proliferation of A431 (A), SK-OV-3 (B), DU 145 (C) and HeLa (D) cells. SRB assay showed the cell viability under the treatment of compound **8** or **9** with the indicated concentrations for 48 h and determined as a percentage of the corresponding control. The data shown represent the means ± SE from 3–5 independent experiments.

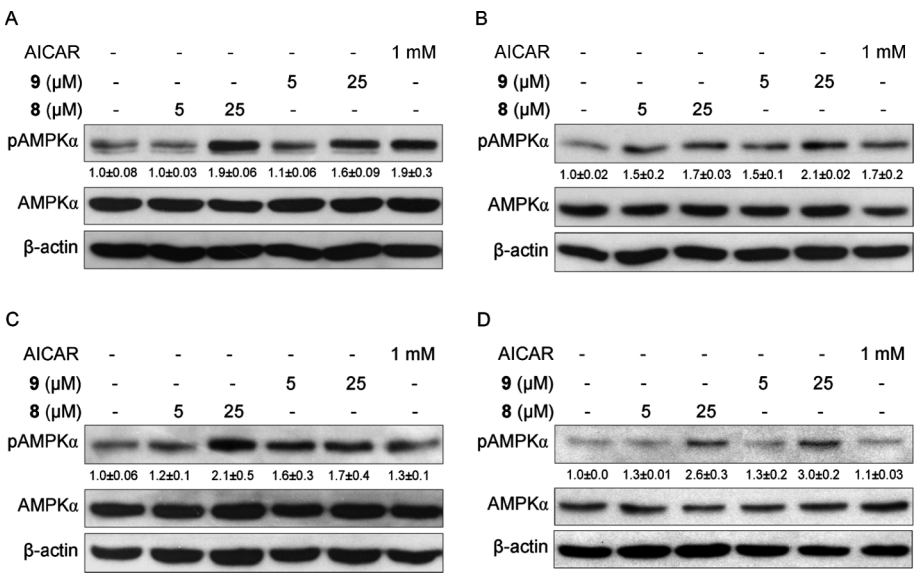


Fig. 3 Compounds **8** and **9**, and AICAR activate AMPK. A431 (A), SK-OV-3 (B), DU 145 (C) and HeLa (D) cells were treated with the indicated concentrations of compounds **8** and **9**, and AICAR, respectively, for 48 h. Western blotting analysis showed the protein levels of phosphorylated AMPKα and total AMPKα. Equal loading was shown by β-actin expression. The numbers below the panels represent the densitometric analysis of AMPKα phosphorylation levels normalized to total AMPKα levels and expressed as fold stimulation over the basal control level (means ± SE, $n = 3$).

Induction of apoptosis was further confirmed by Hoechst 33342 staining. As shown in Fig. 4, compound **9** and AICAR caused morphological changes in cell nuclei (chromatin condensation and DNA fragmentation), suggesting that the inhibition in tumor cell growth by the novel baicalein derivatives might be mediated by the activation of AMPK and the induction of apoptotic cell death.

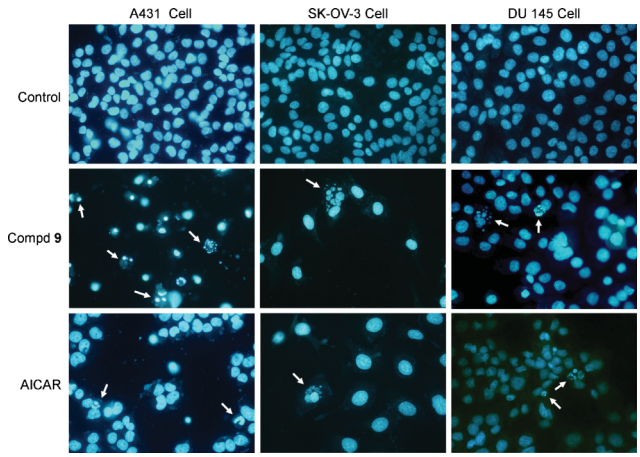


Fig. 4 The effect of compound **9** and AICAR on cell apoptosis measured by Hoechst 33342 staining. A431 (left), SK-OV-3 (middle) and DU145 (right) cells were treated with 25 μM compound **9** or 1 mM AICAR for 48 h. Apoptotic cells with fragmented nuclei (arrows) are seen in the cells treated with compound **9** (middle panel) or AICAR (bottom panel), but not in the control cells (upper panel). Magnification, ×400.

3. Conclusions

In conclusion, we prepared a number of novel baicalein derivatives containing various alkyl and substituted benzyl groups. The cytotoxicities of the synthetic baicalein derivatives were evaluated, and two of the most potent synthetic compounds, **8** and **9**, were determined for their ability for activation of AMPK in A431, SK-OV-3, DU 145 and HeLa cells. The results revealed that the derivatives **8** and **9** were able to activate AMPK by enhancing the levels of phosphorylated AMPKα in A431, SK-OV-3 and DU 145 cells, and particularly in LKB1-deficient HeLa cells, and the compounds demonstrated more potent anti-proliferative effects than baicalein and AICAR. Our findings in this study show that activating AMPK by these novel baicalein derivatives might be an alternative therapeutic approach to exert anti-proliferative functions.

Author contributions

Derong Ding and Baozi Zhang synthesized the compounds. Ying Ma performed the experiments of cell apoptosis. Xin Wang performed HPLC and HRMS analysis. Tao Meng, Hongli Peng and Jingkang Shen co-wrote the paper. Hongli Peng and Jingkang Shen supervised the research.

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